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High-performance liquid chromatographic determination of peroxisomicine A_1 (T-514) in genus *Karwinskia*

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Abstract

A chromatographic method was developed for the T-514 determination in *Karwinskia* leaves, stems and roots. A C_{18} analytical column and a mobile phase consisting of methanol and McIlvaine buffer (pH 3) were used. T-514 was detected using a diode array detector and the chromatograms were recorded at 269 and 410 nm. A linear dependence of a peak area on the T-514 concentration ($r=0.9991$) was obtained in the range of 0.126–12.6 μg/ml. Limits of T-514 quantification (signal-to-noise ratio 10) in plant samples were 126 ng/ml at 410 nm and 28 ng/ml at 269 nm. T-514 was extracted from the plant material with ethyl acetate. Optimal extraction conditions were studied: number of extraction steps, volume of extracting agent and extraction time. The extracts were cleaned up using solid-phase extraction (SPE). SPE recoveries of 99.9% and 98.4% were achieved for the T-514 concentrations of 1.4 μg/ml and 0.26 μg/ml, respectively.

Keywords: Peroxisomicine

1. Introduction

The genus *Karwinskia* (family *Rhamnaceae*) includes 15 kinds of trees and shrubs [1]. Named plants belong among toxic ones [1,2]. High toxicity is caused by the presence of secondary metabolites which were isolated for the first time from the seed endocarp of *Karwinskia humboldtiana* (Roem. and Schut.) Zucc. by Dreyer et al. [3]. The toxins were isolated from *Karwinskia* toxic seeds by the $CHCl_3$ extraction. Four isolated components were defined as anthracenones and identified as: 7-[3′,4′-dihydro-7′,9′-dimethoxy-1′,3′-dimethyl-10′-hydroxy-1′H-naphtho[2′, 3′-*c*′]pyran-5′-yl]-3,4-dihydro-3-methyl-3,8,9-trihydroxy-1(2H)-anthracenone; 3,4-dihydro-3,3′-dimethyl-1′,3,8,8′,9-pentahydroxy(7,10′-bianthracene)-1,9′(2H,10′H)-dione; 7-(2′-acetyl-6′,8′-dimethoxy-3′-methyl-1′-hydroxynaphth-4′-yl)-3,4-dihydro-3-methyl-3,8,9-trihydroxy-1(2H)-anthracenone; and 3,3′-dimethyl-3,3′,8,8′,9,9′-hexahydroxy-3,3′,4,4′-tetrahydro(7,10′-bianthracene)-1,1′(2H,2′H)-dione (Fig. 1). They are, respectively, also named T-544, T-496, T-516 and T-514 due to their molecular masses.

Guerrero et al. [4] published a modification of the method described above in order to simplify the

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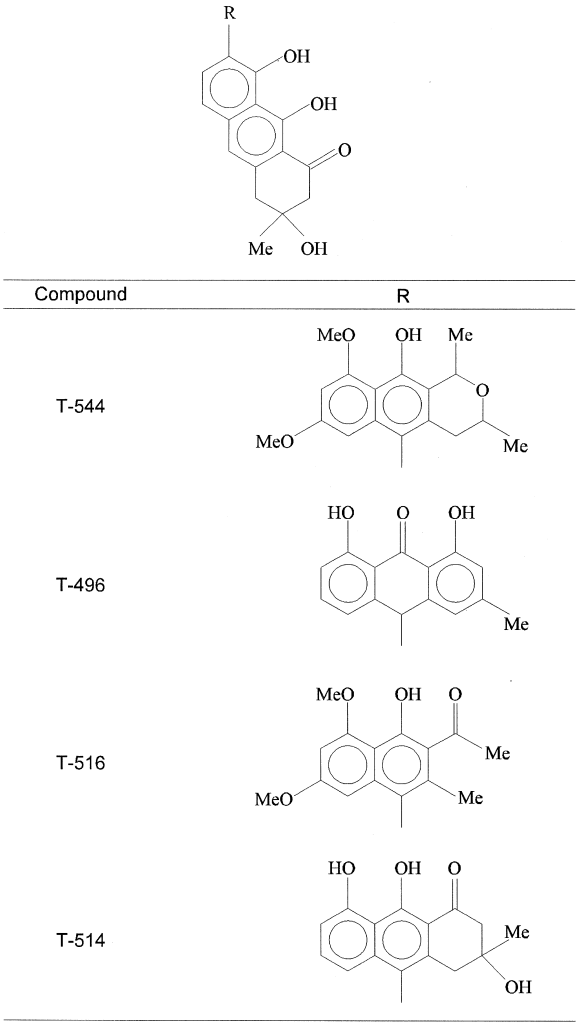

Fig. 1. Chemical structures of T-544, T-496, T-516 and T-514.

procedure of the purification for T-514, as well as for T-544 and T-496.

Because T-514 exhibits selective toxicity in vitro on some tumor cells, it has become a potential antineoplastic agent and intensive research has been started [5]. According to the percentage content of T-514 in different species of genus *Karwinskia*, the greatest interest is concentrated on species *Karwinskia humboldtiana* (Roem. and Schut.) Zucc. and *Karwinskia parvifolia* Rose [6].

Until recently, thin-layer chromatography (TLC) combined with the densitometry has been mostly applied to the determination of T-514 in fruits of *Karwinskia*. Since T-514 has been under preclinical study, the demand for a more sensitive and accurate method has increased.

The first report dealing with the high-performance liquid chromatographic determination of T-514 in seeds from *Karwinskia* appeared in 1996. Salazar et al. [5] described a high-performance liquid chromatography (HPLC) assay for the separation and determination of T-514 and some related anthracenonic compounds in seeds of *K. parvifolia* and *K. humboldtiana*. Liquid extraction with petroleum ether and ethyl acetate (EtOAc) was applied as a fruit preseparation step. Protein precipitation with acetonitrile (MeCN) was recommended by the authors for blood samples spiked with T-514. Extraction recoveries were in the range 82–84%. Although the compounds were separated in 10 min, not all of them were separated with the sufficient chromatographic resolution values necessary for quantitative analysis. Especially, T-514 was not successfully separated from both peaks eluting before and after it.

The aim of the presented work was to develop a simple and complete HPLC method for the determination of T-514 in different parts of *K. humboldtiana* and *K. parvifolia* (leaves, stems and roots) combined with an efficient preseparation technique for the isolation of analyzed compound from the plant matrix.

2. Experimental

2.1. Chemicals and samples

Methanol (MeOH) and acetonitrile (both of chromatography grade) were obtained from Merck (Darmstadt, Germany), EtOAc, citric acid and $Na_2HPO_4 \cdot 12H_2O$ (all of analytical-reagent grade) were from Lachema (Brno, Czech Republic). Standard of T-514 and plants of *K. parvifolia* and *K. humboldtiana* grown in Mexico were obtained from Departamento de Farmacología y Toxicología Facultad de Medicina U.A.N.L. (Montery, N.L., México), plants grown in the Slovak Republic were from the Department of Plant Physiology, Faculty of Natural Science, Comenius University (Bratislava, Slovak

Republic). Growing conditions are described in Refs. [7,8]. Leaves, stems and roots were analyzed just after collection.

2.2. Equipment

An autosampler Basic-Plus Marathon Spark (Emmen, The Netherlands), a HP 1100 system (Hewlett-Packard, Waldbronn, Germany) consisting of a pump with a degasser, a diode array detection (DAD) system and a data station HP ChemStation were used. Separations were carried out using the following chromatographic columns: NovaPak C_{18} (150×3.9 mm; 4 μm) Waters (Milford, MA, USA), the guard column NovaPak C_{18} (20×3.9 mm) Waters, WRP-18 (250×4 mm; 7 μm) Watrex (Bratislava, Slovak Republic), and Nucleosil C_{18} (250×4 mm; 5 μm) Watrex.

The rotary vacuum evaporator RVC-64 (Prague, Czech Republic), shaker T-22 Lověna (Prague, Czech Republic), the Straume mill (Russia), the Dorcus SPE vacuum manifold (Tessek, Prague, Czech Republic), filter paper Filtrak No. 368 (Niederschlag, Germany) and Bakerbond C_{18} (100 mg) solid-phase extraction (SPE) cartridges from Mallinckrodt Baker (Griesheim, Germany) were used for the sample preparation.

2.3. Solutions

A stock solution of T-514 (ca. 100 μg/ml) was prepared in MeOH and stored in a refrigerator at −20°C. Working solutions were prepared by diluting the stock solution with MeOH.

McIlvaine buffer (pH 3) was prepared mixing 795 ml of 0.1 *M* citric acid and 205 ml of 0.2 *M* Na_2HPO_4.

2.4. Chromatographic conditions

Operating conditions for HPLC were: an ambient temperature (23°C), a mobile phase consisting of MeOH–McIlvaine buffer (pH 3) (74:26, v/v), a flow-rate of the mobile phase 0.6 ml/min, DAD at 269 and 410 nm, an injection volume of 20 μl.

2.5. Sample preparation

2.5.1. Liquid extraction

Fresh leaves, stems and roots of *K. humboldtiana* and *K. parvifolia*, were homogenised by grinding and blending in mortar. T-514 was isolated from samples (0.1 g) by three-fold extraction, each time with 5 ml of EtOAc for 30 min. Extraction was done using the laboratory shaker at room temperature (23°C). The extracts were filtered through the filter paper, pooled and evaporated. Dry residues were dissolved in 2 ml of MeCN–deionized water (40:60).

2.5.2. Solid-phase extraction

An SPE cartridge (C_{18}) was conditioned with 3 ml of MeOH and 3 ml of deionized water. A 1-ml sample of the extract was loaded. After washing with 2 ml of MeCN–water (20:80), T-514 was eluted with 4 ml of MeOH–McIlvaine buffer, pH 3 (90:10). The eluent was evaporated and dissolved in 1 ml of the mobile phase.

Extraction recoveries of the SPE procedures were studied at two concentration levels injecting the leaf extracts before and after SPE. Both sample extracts were treated by SPE three times.

3. Results and discussion

The aim of the presented work was to develop a simple and reliable HPLC method for the determination of T-514 in leaves, stems and roots of genus *Karwinskia*.

The following analytical columns were tested: WRP-18 (250×4 mm; 7 μm), Nucleosil C_{18} (250×4 mm; 5 μm) and NovaPak C_{18} (150×3.9 mm; 4 μm). The best chromatographic resolutions of T-514 and peaks eluting before and after the T-514 peak were achieved using the NovaPak C_{18} column.

The mobile phase consisting of MeCN, citric acid and water used in Ref. [5] was not suitable because of a high asymmetry of the T-514 peak. Using a mixture of MeOH and water as a mobile phase in the range 40–100% of MeOH, T-514 did not elute until 60 min. It was necessary to adjust the pH of the mobile phase to a low value. As the acetic buffer could not be used (due to the peak asymmetry), a

McIlvaine buffer consisting of 0.1 *M* citric acid and 0.2 *M* Na_2HPO_4 was also tested. A content of MeOH lower than or equal to 74% was suitable for a sufficient chromatographic resolution of T-514 and coeluting peaks. In the case of analyses of roots, using this mobile phase, an unidentified peak co-eluted with the peak of T-514, therefore it was necessary to use lower content of MeOH (65%). It was not possible to establish one isocratic HPLC system which could be applied to analyses of leaves, stems and roots. Use of a gradient system has not been checked.

T-514 has two absorbance maxima in the UV–Vis spectrum (269 and 410 nm). Since the UV spectra of the neighboring peaks are almost identical with that of T-514, DAD could not ensure peak purity of T-514, however, DAD was used to check if other interferences did not elute together with T-514. Although the molar absorbance coefficient for T-514 at 410 nm is approximately 4.5-times lower than the one at 269 nm, the former wavelength is more suitable because of less number of interfering peaks occurring in the chromatograms of the biological samples.

The linearity of the detector response was tested at eight different T-514 concentrations in the range of 0.126–12.6 μg/ml. Each standard solution was injected three times. A linear dependence of peak area on the T-514 concentration was obtained ($r=0.9991$) with the following linear regression equation, $y=61.8x$ where y is the T-514 peak area (mAU s) and x is the concentration of T-514 solution (μg/ml). All quantitative analyses were performed according to the calibration curve.

Liquid extraction presents the first step of the preseparation procedure of a solid sample, as it is necessary to extract an analyte from the matrix.

The following solvents were tested for the liquid extraction: $CHCl_3$ and EtOAc. A higher extraction yield was the reason for choosing EtOAc. Optimal extraction conditions were studied: number of extraction steps, volume of extracting agent and extraction time. Three parallel samples were prepared from each plant part and all sample extracts were injected three times onto the chromatographic column. Yields of T-514 from *Karwinskia* leaves are listed in Table 1. It is obvious from this table that three extraction steps (each step 30 min, 5 ml of extracting agent/0.1 g of fresh material) are sufficient for the quantitative leaf extraction. The differences between values of 243 and 245 (optimization of number of extraction steps), and 245, 246 and 242 (optimization of extraction time), respectively, are not statistically significant. The difference between 245 and 238 (optimization of extraction agent volume) was caused probably by the differences in losses during the filtration of high volume solution or during the evaporation (losses at flask walls). The similar results were obtained for the stem and root extractions.

As the sample after the liquid extraction still contained a considerable amount of interfering compounds, especially plant pigments, it was advantageous to use another technique for removing the impurities. In our case, SPE was tested and recommended. After washing the C_{18} cartridges with MeCN–water (20:80), T-514 was eluted with MeOH–McIlvaine buffer (pH 3) (90:10). Using SPE, the partial removing of impurities eluting in the first 5 min was achieved, but peaks eluting before

Table 1
Yields and RSD values of T-514 extracted from *Karwinskia* leaves under different extraction conditions ($n=3$)

No. extraction steps	Yield of T-514		Volume of extraction agent (ml)	Yield of T-514		Time of extraction step (min)	Yield of T-514	
	(μg/g)	RSD (%)		(μg/g)	RSD (%)		(μg/g)	RSD (%)
1	210	2.7	2.5	219	5.2	30	245	2.4
2	236	2.0	5	245	2.4	60	246	1.4
3	243	2.1	10	238	2.0	90	242	1.2
4	245	2.4						
5	245	2.4						

and after the T-514 peak did not disappear. It could be supposed that these not identified components are another dimeric anthracenones having similar characteristics as T-514. Their spectra confirmed this fact.

The dependences of the SPE recovery on the eluent volume for two T-514 concentration levels are shown in Fig. 2. It is obvious that the eluent volume of 4 ml MeOH–McIlvaine buffer is sufficient. Extraction recoveries of the SPE procedures ($n=3$) were 99.9% (standard deviation, SD, 1.3%) and 98.4% (SD, 1.0%) for the T-514 concentrations of 1.4 μg/ml and 0.26 μg/ml, respectively. The chromatograms of leaf extracts before and after SPE are illustrated in Fig. 3. T-514 eluted in 13.0±0.3 min and was resolved sufficiently from the other peaks.

Due to the long time of the preseparation procedure and a large number of samples, the analytical column protected by the guard column was used instead of SPE. The T-514 retention time increased to 13.5±0.3 min. The influence of the guard column on the pressure and the peak shape was minimal. Several hundred analyses can be carried out without any change in the chromatographic system.

The chromatograms of *Karwinskia* stem and root extracts using the analytical column protected by the guard column are shown in Figs. 4 and 5. In the case of the root analysis, the retention time of T-514 was

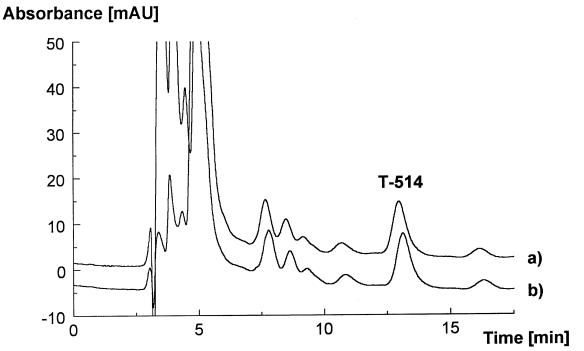

Fig. 3. Chromatograms of leaf extracts: (a) before SPE; (b) after SPE. Chromatographic conditions: chromatographic column, NovaPak C_{18} (150×3.9 mm; 4 μm); mobile phase, MeOH–McIlvaine buffer (pH 3) (74:26, v/v); flow-rate, 0.6 ml/min; DAD at 410 nm; ambient temperature (23°C); injection volume of 20 μl.

56.5±1.0 min as the mobile phase consisted of MeOH–McIlvaine buffer (pH 3) (65:35) (as mentioned above).

Limits of T-514 quantitation (signal-to-noise ratio 10) in plant samples were 126 ng/ml at 410 nm and 28 ng/ml at 269 nm. The repeatability was measured by two-fold injection of three parallel samples and it was calculated as a relative standard deviation (RSD, Table 2).

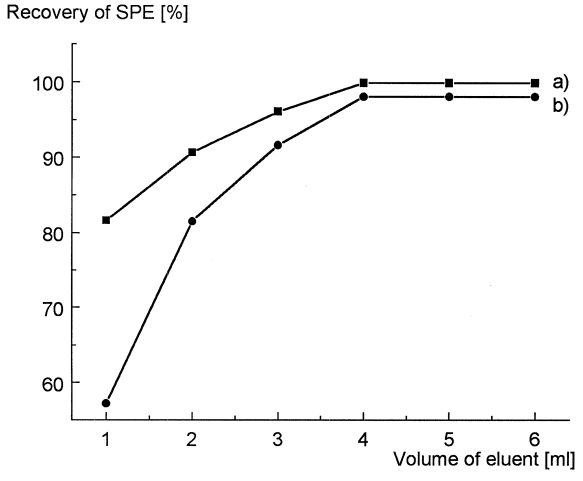

Fig. 2. Dependence of the SPE recovery on the eluent volume: (a) leaf extract, T-514 concentration 1.4 μg/ml; (b) leaf extract, T-514 concentration 0.26 μg/ml.

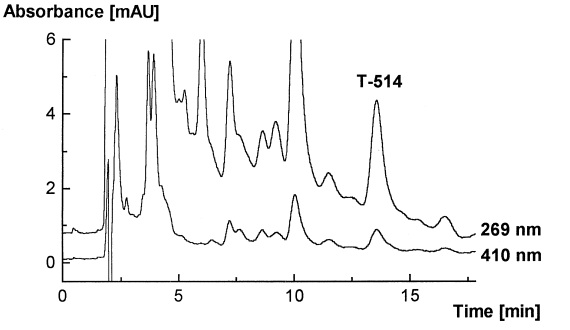

Fig. 4. Chromatogram of the stem extract using the analytical column protected by the guard column. Chromatographic conditions: chromatographic column, NovaPak C_{18} (150×3.9 mm; 4 μm); guard column, NovaPak C_{18} (20×3.9 mm); mobile phase MeOH–McIlvaine buffer (pH 3) (74:26, v/v); flow-rate, 0.6 ml/min; DAD at 269 and 410 nm; ambient temperature (23°C); injection volume of 20 μl.

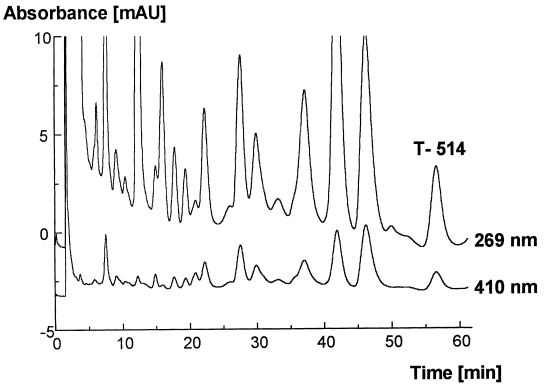

Fig. 5. Chromatogram of the root extract using the analytical column protected by the guard column. Chromatographic conditions: chromatographic column, NovaPak C_{18} (150×3.9 mm; 4 μm); guard column NovaPak C_{18} (20×3.9 mm); mobile phase, MeOH–McIlvaine buffer (pH 3) (65:35, v/v); flow-rate, 0.6 ml/min; DAD at 269 and 410 nm; ambient temperature (23°C); injection volume of 20 μl.

The developed method was applied to the analyses of leaves, stems and roots of *K. parvifolia* and *K. humboldtiana*, respectively, grown under different growing conditions (one-, two- or three-year-old plants). Detailed growing conditions and their possible influences on the T-514 contents have been published in Refs. [7,8].

Table 2
Yields and RSD values of T-514 extracted from the different parts of *Karwinskia parvifolia* and *Karwinskia humboldtiana* (n=3)

	K. parvifolia		*K. humboldtiana*	
	Yield of T-514		Yield of T-514	
	(μg/g)	RSD (%)	(μg/g)	RSD (%)
Leaves	77.7	2.6	68.2	3.4
Stems	11.7	6.8	7.5	5.2
Roots	15.5	2.5	6.1	4.5

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